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Detection of advanced oxidation protein products in patients with chronic kidney disease by a novel monoclonal antibody

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Abstract

Advanced oxidation protein products (AOPP) as a biomarker of oxidative stress has been demonstrated in chronic kidney disease (CKD) patients; however, current methods to detect the accumulation of AOPP in serum and in tissues are limited and unreliable. This study generated a monoclonal antibody (mAb) designated 3F2, that reacts specifically with hypochlorous acid (HOCl)-modified proteins, but not with the native forms or with other types of oxidative modifications. Notably, mAb 3F2 recognizes the AOPP deposited in renal tissues of AOPP-treated rats and of patients with different kinds of CKD. Moreover, this mAb can almost completely inhibit the production of reactive oxygen species in RAW264.7 cells induced by AOPP ($p < 0.001$). In conclusion, mAb 3F2 can be used to detect AOPP specifically in serum and in tissues, and this antibody can potentially provide an important tool and new insight into research on diseases related to oxidative stress.

Keywords: *Advanced oxidation protein products (AOPP), monoclonal antibody (mAb), chronic kidney disease (CKD), oxidative stress, immunoassay*

Abbreviations: *AOPP, advanced oxidation protein products; CKD, chronic kidney disease; mAb, monoclonal antibody; HOCl, hypochlorous acid; HD, haemodialysis; LDL, low-density lipoprotein; MSA, mouse serum albumin; HSA, human serum albumin; OD, optical density; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel; ROS, reactive oxygen species; RT, room temperature.*

Introduction

The prevalence and incidence of chronic kidney disease (CKD) have progressively increased worldwide [1,2]. An unfortunate aspect of chronic kidney disease will eventually progress to end-stage renal disease. The mechanisms underlying the progression of chronic kidney disease remain elusive. We and others have identified advanced oxidation protein products (AOPP) as emerging new pathological factors for the development of chronic kidney disease [3–7]. AOPP are the dityrosine-containing and cross-linking protein products formed during oxidative stress by the reaction of serum protein with chlorinated oxidants, such as hypochlorous acid (HOCl) [3]. AOPP are also formed *in vitro* when serum albumin is exposed to HOCl. Initially, AOPP were considered a marker of oxidant-mediated protein damage in some diseases [8–11]. Our previous studies found that chronic accumulation of AOPP promotes inflammation in diabetic and non-diabetic kidneys and worsens inflammation [12] and oxidative stress in arteries in a hyperlipidemia model [4]. These data suggest that the oxidized proteins, by themselves, may contribute to the progression of chronic kidney disease as well as its related complications. Therefore, monitoring accumulation of AOPP in serum, plasma or renal tissue represents

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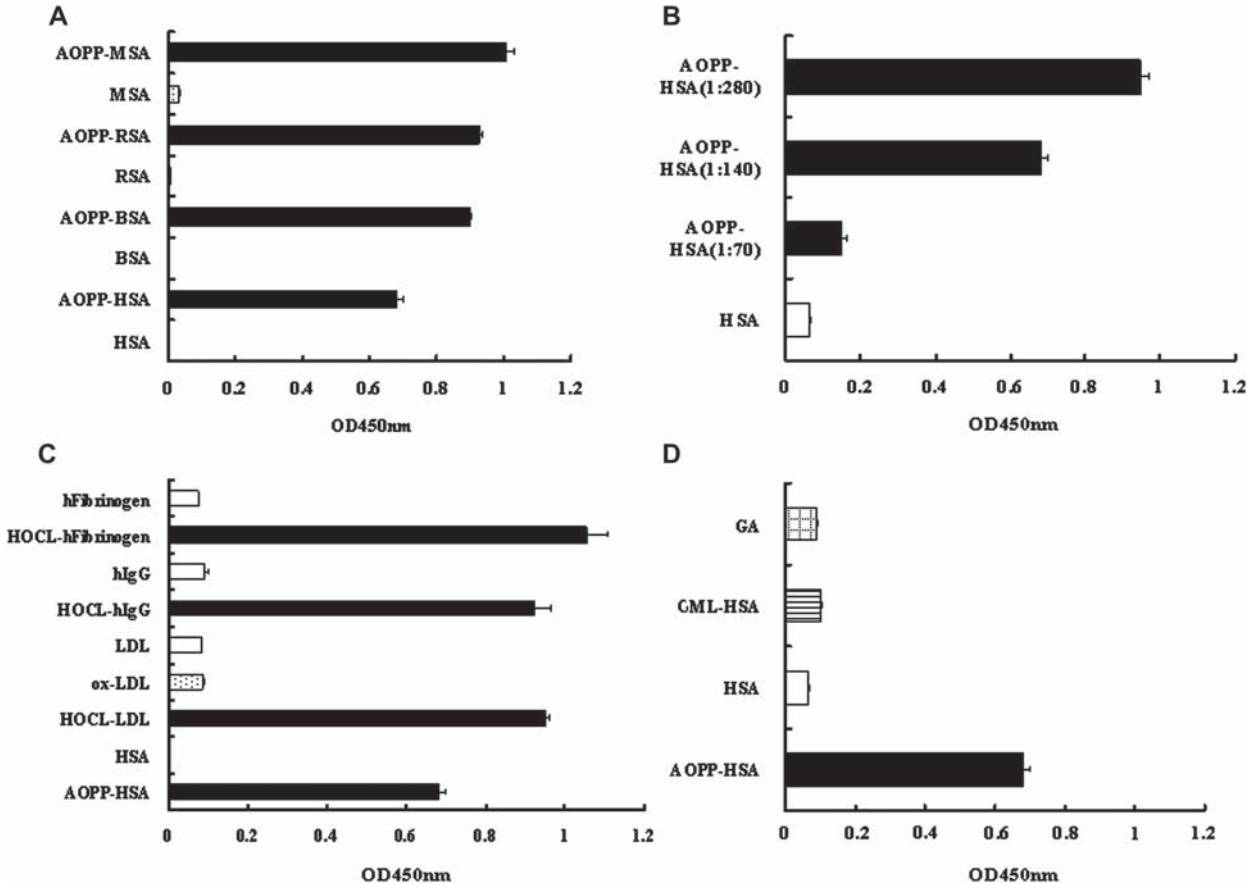

Figure 1. The specific binding of mAb 3F2 to HOCl modified proteins was detected by indirect ELISA. A. The binding of 3F2 to AOPP-albumin from different genera. (The molar ratio of serum albumin to HOCl is 1:140), native albumin as negative control. MSA: mouse serum albumin, RSA: rabbit serum albumin, BSA: bovine serum albumin, HSA: human serum albumin. B. The binding of 3F2 to AOPP-HSA oxidized at different molar ratios (1:70, 1:140, and 1:280). C. Binding of 3F2 to HOCl-HSA, HOCl-IgG, HOCl-fibrinogen and HOCl-LDL (the molar ratio of the protein to HOCl is 1:140), as well as Cu^{2+} oxidized human LDL (ox-LDL). D. Binding of 3F2 to glycosylated modified HSA which includes glycolaldehyde-HSA (GA-HSA) and Carboxymethyl-HSA (CML-HSA). AOPP-HSA and HSA were used as positive and negative control, respectively.

a new strategy for the prediction of progression of chronic kidney disease as well as for evaluating other conditions related to oxidative stress such as metabolic syndrome.

Serum or plasma AOPP levels are commonly determined by spectrophotometry to measure the absorbance at 340 nm under acidic conditions and this assay is calibrated with chloramine-T equivalents [3]. However, several factors such as high triglycerides [13], fibrinogen in serum [14] and freeze/thaw cycles can interfere with sample turbidity, thereby affecting the accuracy of the measurement. Another limitation of this method is that it is not suitable for detecting or determining the localization of AOPP in tissues.

This study would be aimed to generate monoclonal antibody (mAb) against AOPP for potential clinical applications. Previous studies have shown that HOCl can readily modify amino group side chains of proteins, leading to protein cross-linking, aggregation and fragmentation [15]. In our work, therefore, the special epitopes formed by HOCl-modified amino acids would be used to induce the production of antibodies against AOPP and the mAb would be characterized and applied primarily in detection of AOPP in serum and renal tissue.

Materials and methods

Blood collection and serum isolation

Blood samples taken from patients with maintenance haemodialysis (HD) ($n = 28$) were drawn from a vein just before the start of the haemodialysis session. Blood samples of healthy donors ($n = 25$) were taken from volunteers in our university. All patients and healthy subjects provided informed consent. Blood (5 ml) was collected in standard sterile polystyrene vacuum tubes. Following centrifugation (600 g $\times$ 10 min) the serum was stored at -70°C until use.

Preparation of AOPP-protein

HOCl oxidative-albumin from different genus, immunoglobulin G (IgG), human fibrinogen and low-density lipoprotein (LDL) were prepared as described previously [3]. Briefly, serum albumin, IgG,

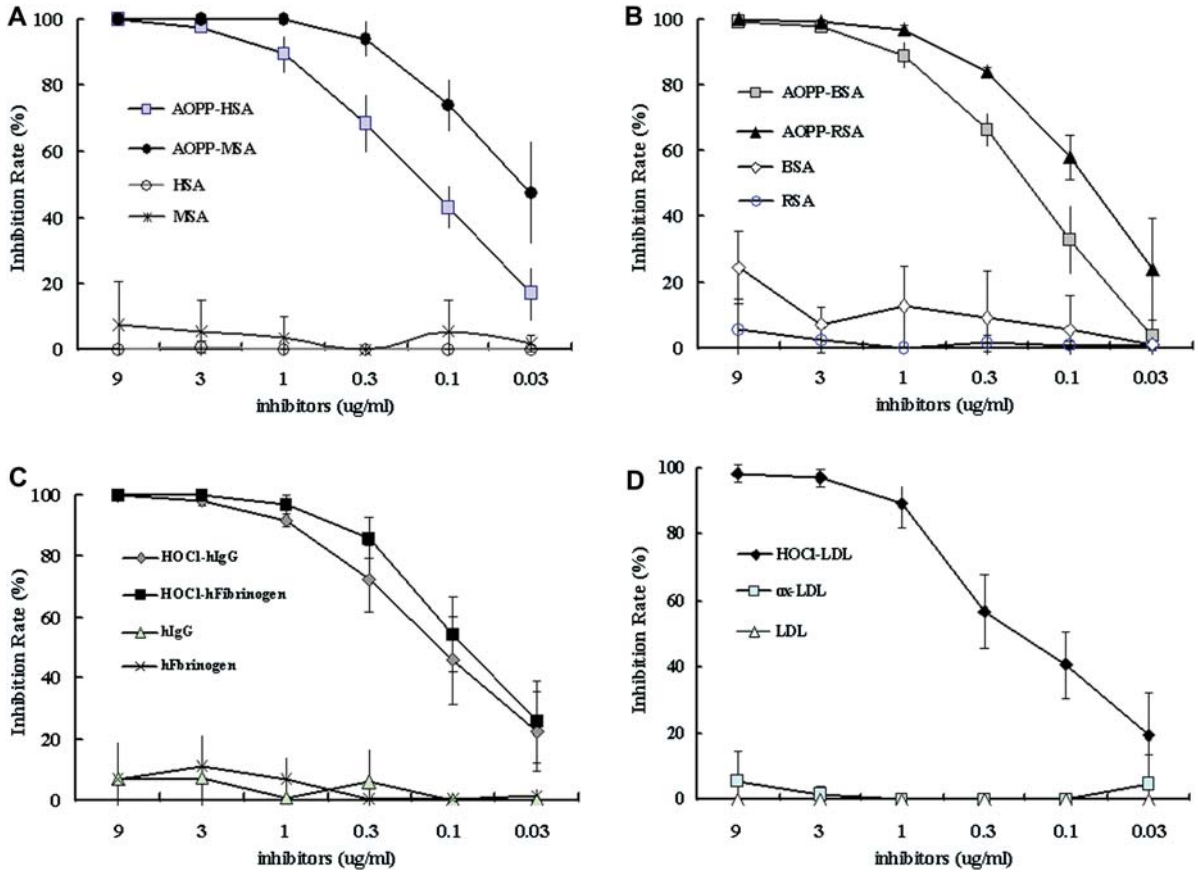

Figure 2. Inhibition of different HOCl-modified proteins/LDL on binding of mAb 3F2 to AOPP-HSA was analyzed by competitive ELISA. The bound of biotin-labeled 3F2 to AOPP-HSA was decreased in the presence of increasing concentration of HOCl-oxidative albumin from different genera (A and B), HOCl-modified human IgG/fibrinogen (C) and HOCl-modified LDL (D), respectively. Native proteins, LDL and Cu^{2+} oxidized human LDL (ox-LDL), which do not bind to 3F2, indicate the background. The inhibition was calculated as a percentage of the absorbance of 3F2 without any HOCl-oxidative proteins/LDL.

fibrinogen (all obtained from Sigma Aldrich, St. Louis, MO) and LDL were treated with HOCl (Fluka, Buchs, Switzerland) at a 1:140 molar ratio for 30 min followed by overnight dialysis against PBS to remove free HOCl. Samples were passed through a Detoxi-Gel column (™ Endotoxin Removing Gel, Thermo Scientific, Pierce, Rockford, IL) to remove contaminated endotoxin. Residual endotoxin was measured with the amebocyte lysate assay kit (Sigma) and was below 0.025 EU/ml. AOPP content was expressed in chloramine-T equivalents (μmol/L) as described previously [3]. Briefly, 200 μl of the sample was added to a 96-well plate (Corning Costar, Corning, NY) and mixed with 20 μl of acetic acid. In standard wells, 10 μl of 1.16 mol/L potassium iodide (Sigma) was mixed with 200 μl of chloramine-T solution (Sigma) followed by the addition of 20 μl of acetic acid. The absorbance of the reaction mixture was immediately read by a microplate reader (BioRad-550) at 340 nm.

Generation of monoclonal antibodies

Equal volumes of AOPP-mouse serum albumin (AOPP-MSA) (100 μg/mouse) were admixed with Imject Alum Adjuvant (Pierce) or emulsified with Complete Freund's adjuvant (Sigma) and injected subcutaneously into 6-week-old BALB/c female mice (Experimental Animal Centre of Southern Medical University, Guangzhou, China). Mice were boosted with the same dose of AOPP-MSA by intraperitoneal injection five times at 2-week intervals. The animals were housed in individual cages under constant temperature and humidity on a 12-h light/dark cycle. Food and water were provided *ad libitum*. Protocols for animal experimentation and maintenance were approved by the Animal Ethics Committee at our institute and carried out in accordance with institutional guidelines. The animals were sacrificed 5 days after the final boost without adjuvant. Splenocytes were fused at a 4:1 ratio with NS-1 mouse myeloma cells by 50% PEG 4000 to generate hybridomas. The anti-AOPP antibodies in the hybridoma culture supernatants were screened by indirect ELISA with AOPP-human serum albumin (AOPP-HSA) and HSA as coated antigen. Only the hybridoma cells that secreted antibodies against AOPP-HSA but not against HSA were cloned by successive limiting dilution. For large-scale preparation of mAbs, BALB/c mice, which were pre-treated with 0.5 ml of

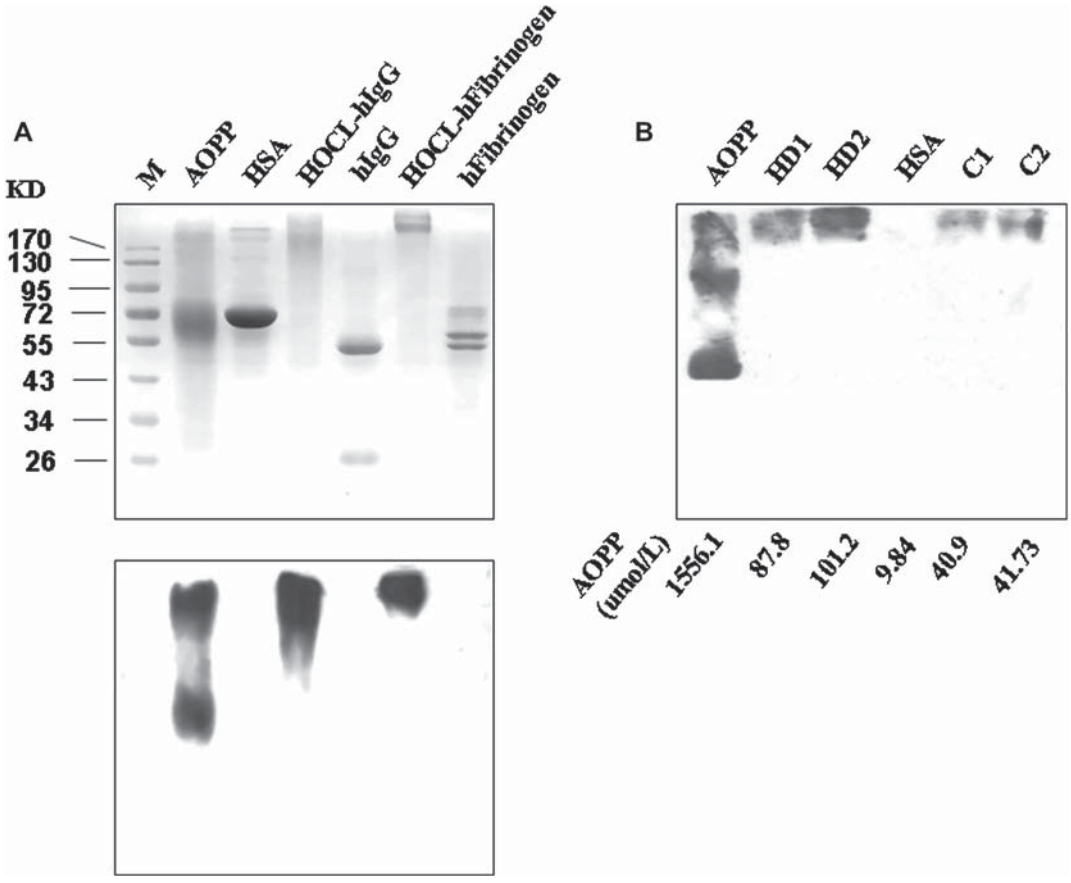

Figure 3. Western Blot analysis HOCl modified proteins in serum. A. (Upper) Proteins treated with or without HOCl under reducing with 100 mM DTT in SDS-PAGE stained with Coomassie bright blue. (Lower) mAb 3F2 probed with above proteins transferred to PVDF membrane. B. Detection of AOPP in human serum by Western Blot based on native PAGE. Serum samples from two hemodialysis patients (HD1, HD2) and two healthy donors (C1 and C2, 30-40 years old) were fractioned on native PAGE and probed with 3F2. HOCl-HSA (AOPP) and HSA were used as positive and negative controls, respectively. The result of one of experiments is presented. Corresponding AOPP levels (μmol/L) expressed by Chloramines-T equivalent values at the bottom of each lane in B.

incomplete Freund's adjuvant (Sigma), were intraperitoneally injected with hybridoma cells (1×10^6 cells/mouse). The ascites were purified by affinity chromatography using HiTrap protein G HP (GE Healthcare Bioscience, Piscataway, NJ). The mAb isotypes were determined using a Monoclonal Antibody Isotype Kit (Thermo Scientific, Pierce).

Indirect ELISA

The 96-well microplates were coated with 50 μl per well of the oxidized or native forms of various proteins at a concentration of 5 μg/ml in coating buffer (0.05 M bicarbonate buffer, pH 9.5) at 4°C overnight and were blocked with 0.25% casein in 0.01 M pH 7.4 phosphate buffered saline (PBS) containing 0.1% Tween 20 (PBS-T) at 37°C for 2 h. After washing five times with PBS-T, 50 μl per well of the diluted hybridoma supernatant or 5 μg/ml purified mAb were added and incubated at 37°C for 30 min and were washed again. Then, the microplates were incubated with a horseradish peroxidase (HRP) labelled rabbit anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 37°C for 30 min and were subsequently washed thoroughly. Colour reactions were developed using tetramethylbenzidine (TMB) with hydrogen peroxide (H_2O_2) as a substrate solution and stopped with 2 M sulphuric acid. The optical density (OD) values were read at 450 nm using a microplate reader (BioRad 550).

Competitive ELISA

The 96-well plates were coated with 100 μl per well of AOPP-HSA at a concentration of 0.5 μg/ml in coating buffer at 4°C overnight. The plates were blocked as described above. After five washes, simultaneous 0.5 μg/ml biotin-labelled 3F2 in the presence of inhibitors (including HOCl-oxidative albumin from different genera, human IgG, fibrinogen and LDL) at indicated concentrations (50 μl per well, respectively) were added into wells and incubated at 37°C for 30 min. Plates were washed thoroughly, peroxidase conjugated streptavidin (Jackson Immuno Research Laboratories) was added and incubated at 37°C for 30 min. The colour reaction was performed as above.

Polyacrylamide gel electrophoresis (PAGE) and Western Blot

The oxidized and native proteins mixed with sample buffer containing dithiothreitol (DTT) as reducing reagent were loaded on sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). Human serum samples and oxidized HSA (AOPP) were fractioned on native PAGE without SDS and DTT, both of which were prepared in 3.9% stacking and 12% separating gel. Then, the proteins in the gel were transferred to polyvinylidene difluoride membranes (PVDF, Millipore, Billerica, MA) and were subsequently blocked with 5% skim milk at room temperature (RT) for 2 h. The membranes were probed with 0.5 μg/ml mAbs in PBS-T containing 5% skim milk at RT for 1 h and were washed five times with PBS-T. The membranes were incubated with HRP-labelled goat anti-mouse IgG (Jackson Immuno Research Laboratories) at RT for 1 h. The signal was detected by the Novex ECL kit (Invitrogen, Shanghai, China). AOPP levels in serum samples were expressed in μmol/L of chloramines-T equivalents.

Preparation of kidney tissue sections

AOPP-treated rats were generated as described previously [6]. In brief, male Sprague-Dawley rats with the initial weight of 180–200 g (Southern Medical University Animal Experimental Center, Guangzhou, China) were bred under standard conditions. The rats were divided into two groups ($n = 10$ in each group) and received daily intravenous injections of either PBS or AOPP-rat serum albumin (100 mg/kg per day). The rats were sacrificed at the end of the 12th week. The kidney tissues were collected after perfusion with 50 ml of ice-cold normal saline (0.9% NaCl). Human renal tissue sections were obtained by the clinical biopsy of patients with different types of kidney disease, who received renal biopsy for diagnosis (diabetic nephrology, $n = 10$; IgA nephropathy, $n = 35$; membranous nephropathy, $n = 10$) or from tumour-free parts of nephrectomy ($n = 3$). All patients were provided with informed consent.

Immunohistochemistry

Immunohistochemistry was performed on 5-μm sections of paraffin-embedded tissues. After deparaffinage, the antigen in citrate buffer (0.01 mol/L, pH 6.0) was restored in the microwave. Endogenous peroxidase, which can adversely affect staining, was eliminated with 0.3% H_2O_2-methanol at RT for 20 min; it was then blocked with 10% goat serum. The tissue sections were incubated with mAb 3F2 (4 μg/ml) or normal mouse IgG1 (the matched isotype) as control at RT for 1 h. After three washes, the sections were incubated with HRP-conjugated goat anti-mouse IgG (EnVision^{TM} Detection Kit, DAKO, Glostrup, Denmark) at RT for 30 min. The colour reaction was developed with 3,3-diaminobenzidine (DAB) solution and subsequently counterstained with haematoxylin. Specific blocking test on tissue section from AOPP-treated rats were performed by adding HOCl-modified rat serum albumin or native albumin mixed with 3F2.

Determination of ROS produced by monocytic RAW264.7 cells

Murine monocytic RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (all of from Hyclone), 100 U/ml penicillin (Gibco) and 100 U/ml streptomycin (Gibco) at 37°C in 5% CO_2. The cells were collected and 2×10^5 cells/well were plated into 96-well microplates for overnight incubation at 37°C in 5% CO_2, then deprived of serum for additional 2 h before the experiment. The levels of intracellular ROS were determined by using an oxidation-sensitive fluorescent probe, 2'7'-dichlorofluorescin diacetate (DCFH-DA, Sigma), which was converted to dichlorofluorescein (DCF) by ROS within cells. RAW264.7 cells were treated with 100 μg/ml AOPP and 20 μg/ml 3F2 or non-immune mouse IgG (mIgG) and incubated for 3 h at 37°C; and cytosolic Cu/Zn superoxide dismutase (c-SOD) (Sigma) was used as a control, inhibiting the production of ROS. Then, 2 μM DCFH-DA was added to the wells for 20 min at 37°C in the dark. The cells were washed five times with PBS. The fluorescence intensity of the cell suspensions was detected by Perkin Elmer wallac 1420 at an excitation wavelength of 500 nm and emission wavelength of 530 nm [16].

Statistical analyses

Statistical analyses were conducted with SPSS 12.0 software (SPSS, Chicago, IL). All the data are presented as mean ± SD. Variable group differences were determined by one-way ANOVA. Significance was defined as $p \leq 0.05$. All experiments were repeated three times.

Results

Generation of a monoclonal antibody against AOPP

AOPP prepared by HOCl-treated MSA was identified by determining the chloramines-T equivalents. AOPP were at a concentration of 4.5 ± 0.5 nmol/mg in HOCl-treated MSA and only 0.2 ± 0.02 nmol/mg in untreated MSA. The isotype for all of the anti-AOPP antibodies generated from the mice immunized with the AOPP-MSA emulsified with complete Freund's

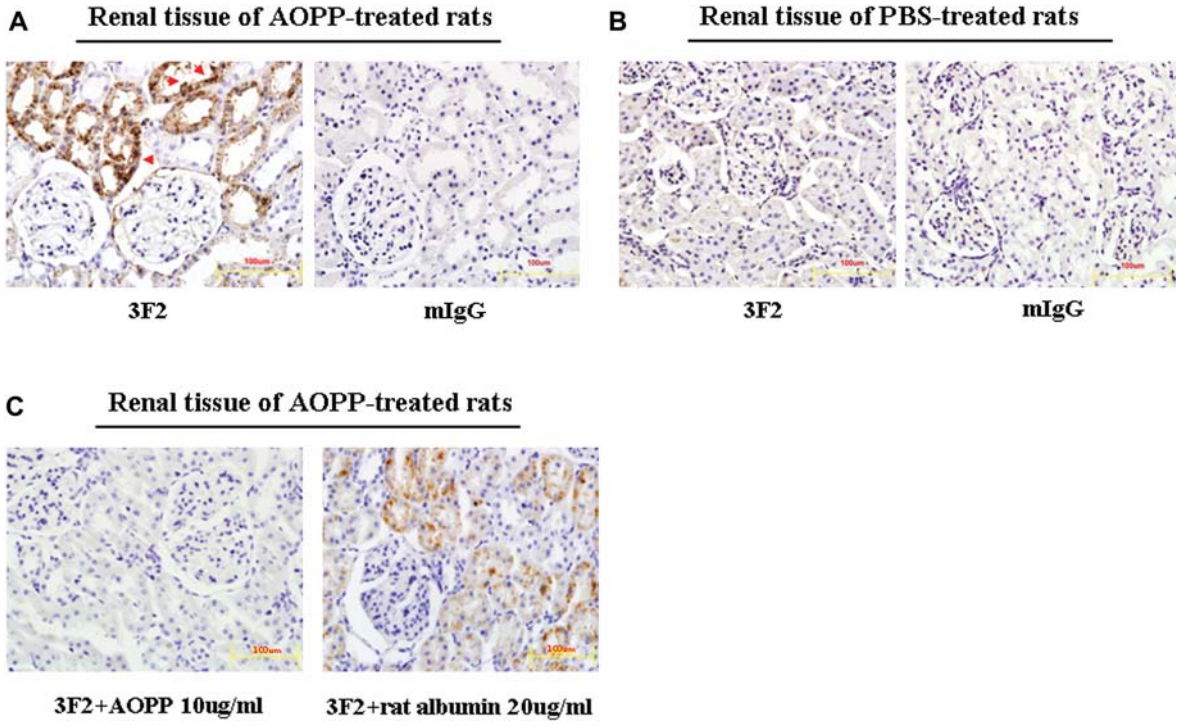

Figure 4. AOPP accumulation in renal tissues of rat models was determined by immunohistochemistry. The location of AOPP in renal tissues of rats treated with AOPP-rat serum albumin (A) or PBS as control (B) was detected by mAb 3F2 (left). Non-immune mouse IgG (right) was used as isotype control. C. Exogenous AOPP blocks the detection of AOPP accumulated in renal tissues. Renal tissues of AOPP-treated rat were incubated with mAb 3F2 in presence of 10 μg/ml AOPP (left) or 20 μg/ml rat serum albumin (right), then the bound of 3F2 to AOPP in tissues were measured by reaction with HRP conjugated goat anti-mouse IgG described in Materials and Methods. The result of one of experiments is presented. The arrows point out AOPP-staining. Bar =100 μm.

adjuvant is IgM, an inappropriate isotype to develop a detecting system for AOPP. Only several strains of mAbs generated from the mice immunized with the AOPP-MSA that had been mixed with Alum adjuvant were IgG1 antibodies, one of which was designated as 3F2 with κ light chain. The mAb 3F2 from ascites with titer 1.0×10^{-7} (as determined by indirect ELISA) was purified by chromatography using a protein G column.

The specificities of 3F2 to HOCl-modified proteins

The binding specificities of the mAb were identified by indirect ELISA, in which the modified or native forms of different proteins were coated on microplates as antigens. The results in Figure 1A show that mAb 3F2 specifically recognized HOCl-oxidative albumin from different genera (mouse, rabbit, bovine and human) but not the native forms. The binding activity of 3F2 increased in parallel with the intensity of HOCl-oxidation (Figure 1B), indicating that 3F2 binds to albumin with different degrees of oxidation. In addition, mAb 3F2 also reacted with HOCl-modified LDL (HOCl-LDL), HOCl-IgG and HOCl-fibrinogen, but not oxidized LDL (ox-LDL) generated by Cu^{2+}, a non-physiological agent (Figure 1C). Moreover, mAb 3F2 did not react with glycosylated proteins, such as carboxymethyl-HSA (CML-HSA) or glycolaldehyde-HSA (GA-HSA) (Figure 1D), that are also formed under oxidative stress in patients with CKD [17], although CML-HSA and GA-HSA were also made with human albumin in this study. Collectively, these results in Figures 1A–D indicate that mAb 3F2 specifically recognizes the AOPP epitope on HOCl-oxidative proteins.

To verify the specificity of the binding between 3F2 and HOCl modified proteins/LDL, the competitive ELISA was performed. The results show that the binding of 3F2 with AOPP-HSA was inhibited in a dose-dependent manner by HOCl-modified proteins from different genera (Figures 2A and 2B), HOCl modified IgG, fibrinogen (Figure 2C) as well as LDL (Figure 2D), whereas this binding was not influenced by native proteins (Figures 2A–C) and Cu^{2+} oxidative LDL (Figure 2D). These results indicated that 3F2 recognizes HOCl oxidative motif specifically.

mAb 3F2 recognizes AOPP in human serum

We next measured whether mAb 3F2 could specifically detect human serum AOPP using Western blot analysis. Figure 3A (upper panel, SDS-PAGE under reducing condition) shows that all of HOCl-modified plasma components such as albumin, IgG and

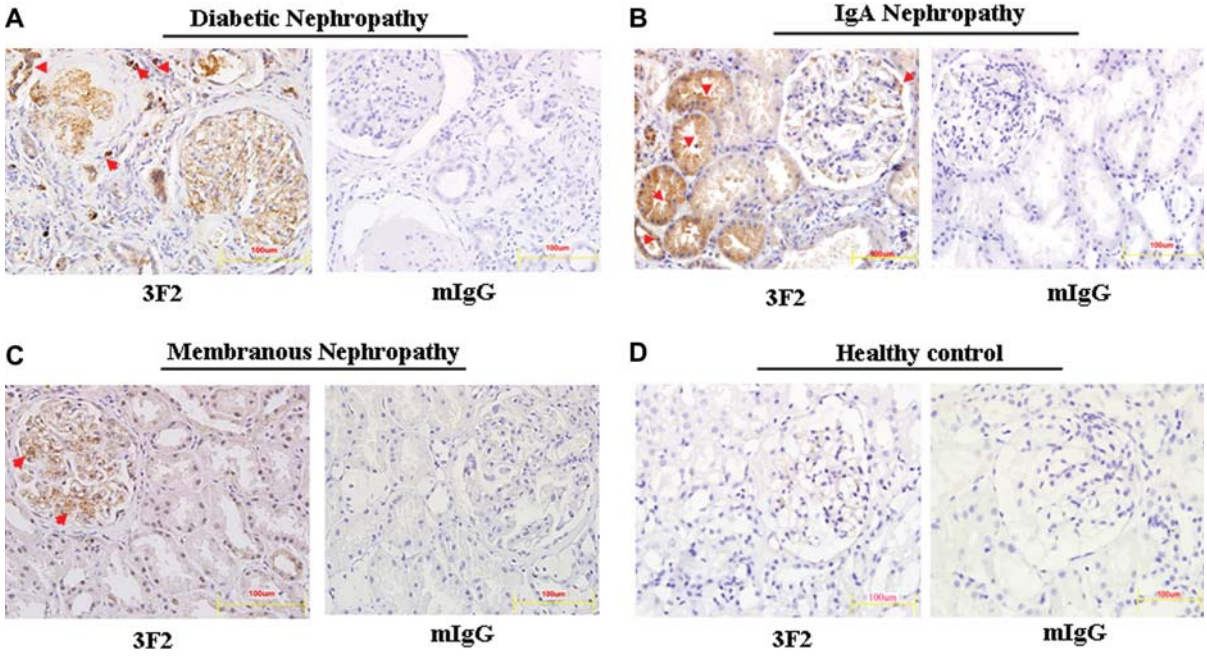

Figure 5. AOPP accumulation in renal tissues from different CKD patients was determined by immunohistochemistry. The renal tissues of patients with diabetic nephropathy (A), IgA nephropathy (B) and membranous nephropathy (C) were immunostained with mAb 3F2 (left) or non-immune mouse IgG (right) as described in Materials and methods. No staining was seen in control sample (D). The result of one of experiments is presented. The arrows point out AOPP-staining. Bar =100 μm.

fibrinogen migrate as a smear, which is similar with the previous work [18]. This phenomenon suggests that the smear may be attributed to protein cross-linking, aggregation or fragmentation induced by HOCl-oxidation. The results in Figure 3A (lower panel) show that mAb 3F2 only binds with HOCl modified HSA, IgG and fibrinogen, but does not bind with native HSA, IgG and fibrinogen; and the major bands recognized by mAb 3F2 are located in part with higher molecule weight (MW), indicating that AOPP in human serum can form aggregate. Unlike a clear difference between artificially prepared AOPP and native proteins, the AOPP was also shown in healthy controls by mAb 3F2 using Western blot based on native PAGE (Figure 3B); however, the difference of AOPP levels between HD patients and healthy controls was distinguishable. Moreover, the reaction with mAb 3F2 in HD patients and healthy controls were coincident with AOPP measured by chloramine-T equivalents (Figure 3B).

Detection and localization of AOPP in renal tissues by immunohistochemistry

Immunohistochemistry with mAb 3F2 was used to detect AOPP in renal tissue from model rats and patients with CKD. The accumulation of AOPP was found in renal tubules of AOPP-treatd rats by 3F2, but not by IgG matched isotype control (Figure 4A); whereas sections from rats treated with PBS did not show any specific colour reaction (Figure 4B). Moreover, the staining in tubules with 3F2 was completely blocked by exogenous AOPP of 10 μg/ml, but not by native albumin of 20 μg/m (Figure 4C). These results suggest that the immuno-staining based on 3F2 was specific for AOPP. The results in Figure 4 also indicate that 3F2 can recognize the common epitope on HOCl-modified proteins from tissues of different genera.

Consequently, we detected the renal tissue sections from patients with different CKD. As presented in Figure 5, a significant accumulation of AOPP with different distribution patterns was observed in the renal tissue of patients with diabetic nephropathy (Figure 5A), IgA nephropathy (Figure 5B) and membranous nephropathy (Figure 5C). AOPP staining was predominantly localized to the mesangium, the glomerular basement membrane (GMB) from tissues of diabetic nephropathy and membranous nephropathy; pronounced colour reaction was also observed in renal tubular epithelial cells and in the mononuclear cells infiltrated into interstitium in samples from diabetic nephropathy and IgA nephropathy. The sections from control sample, however, did not show any staining by mAb 3F2 (Figure 5D).

mAb 3F2 inhibited the production of ROS by RAW264.7 cells stimulated with AOPP

Previous studies demonstrated that AOPP could induce monocyte respiratory burst [19]. In the present work, AOPP-induced over-production of ROS in cultured RAW264.7 cells was almost completely inhibited by mAb 3F2 (Figure 6), which suggests that

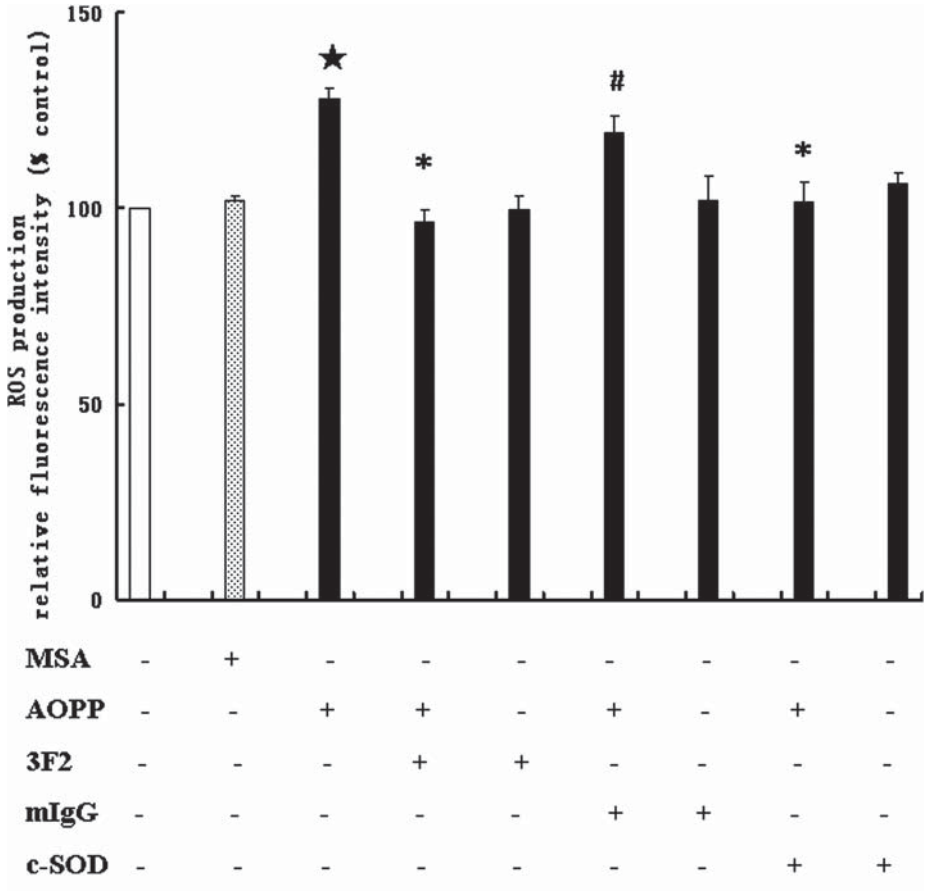

Figure 6. Inhibition of mAb 3F2 on ROS production induced by AOPP. RAW264.7 cells were stimulated with 100 μg/ml AOPP-MSA with or without 3F2 (20 μg/ml) for 3 hours and followed by incubation with 2 μM DCFH-DA. ROS production was assayed by measuring the fluorescence of dichlorofluorescein (DCF). Each value represents the mean±SD. ★$p<0.001$, in comparison with the control group. *$p<0.001$, in comparison with the AOPP-treated group. #$p>0.05$, in comparison with AOPP-treated group.

mAb 3F2 can specifically block the pro-inflammatory activity of macrophage stimulated with AOPP.

Discussion

It has been generally accepted that immunoassay and immunohistochemistry are very specific and reliable techniques for the diagnosis of and pathogenesis research on many diseases. In atherosclerosis, diabetes and CKD, oxidation and glycosylation of proteins under stress, ageing or metabolic abnormalities have been emphasized as disease markers, and AOPP have been described as reliable biomarkers for the evaluation of oxidation induced by HOCl from the 1990s [3]. The detection and localization of AOPP in human serum and tissues with a specific antibody would help to elucidate the pathological effect of AOPP and to develop a new therapeutic strategy on these diseases. For generation of a useful anti-AOPP mAb, immunogen and adjuvant should be considered as major factors that influence the specificity and Ig sub-class. To distinguish from the conventional protocol, we chose the HOCl-modified homologous albumin (MSA) as an immunogen to avoid provoking a stronger antibody reaction against the epitope on heterologous albumin (such as HSA) rather than the AOPP group. In addition, the application of this homologous immunogen also notably decreased the cross-reactivity with the native forms of the proteins, especially in screening of the monoclonal antibodies with heterologous AOPP-albumin as a coating antigen. We developed successfully a monoclonal antibody, 3F2, that recognizes a common epitope on HOCl-modified proteins (as shown in Figures 1–3A).

The AOPP content in serum of HD patients markedly increased compared with that of healthy donors, which showed an obvious parallel between AOPP content detected by Western blot and calibrated by chloramine-T equivalents (Figure 3B). These results indicate the advantages and application of mAb 3F2 in quantitative analysis of plasma or serum AOPP. Witko-Sarsat et al. [3] demonstrated that AOPP was composed of a high molecular-weight (~ 670 kD) fraction and a low-molecular weight (~ 50 kD) fraction, our work indicates that mAb 3F2 mainly recognizes the high-molecular-weight AOPP fraction (Figure 3). Additionally, the reactivity of mAb 3F2 with AOPP could not be abolished by reducing with thiol reductants such as DTT (Figure 3A, lower

panel) or β-mercaptoethanol (data not shown), which suggests that there is an irreversible HOCl-modification on proteins [15,20,21]. This irreversible protein modification may contribute to inflammation, especially in lesions of kidney tissue [5–7]. However, this irreversible protein modification that is resistant to reductants seems to be unstable in the fluid phase or in serum samples, since a degradation smear below MW 67 kDa (molecular weight of HSA) was found by SDS-PAGE (Figure 3A upper). The above results by Western blot can characterize the AOPP and mAb 3F2. However, the best quantitive technique for serum AOPP should be ELISA that is convenient and simple. We tried to set up sandwich ELISA with several antibody pairs including two monoclonal antibodies (another mAb 4C5, data not shown) or monoclonal antibody paired with polyclonal antibody. Unfortunately the sandwich ELISA with different antibody combination only could detect artificial AOPP specifically and sensitively, but could not detect the natural AOPP in serum, which would be attributed to diassociation of aggregate or polymer in serum AOPP under the thoroughly washing in ELISA procedure. Therefore, we have started a new attempt to develop a more sensitive technique to measure serum AOPP by time resolved fluoroisnmunoassay (TRFIA) which would be expected to catch monomer AOPP dissociated from polymer and also tried to develop an immunoturbidimetric assay to avoid dissociation of polymer AOPP.

Concerning the application of mAb 3F2, the most notable would be to recognize the AOPP deposition in kidney lesion tissue from model rats and patients with different CKD including diabetic nephropathy, IgA nephropathy and membranous nephropathy (Figures 4 and 5). Moreover, mAb 3F2 can accurately localize the distribution of AOPP in the mesangium, glomerular basement membrane (GMB) and renal tubular epithelial cells. Malle et al. generated mAb 2D10G9 [22] and 10A7H9 [23] in the supernatant of hybridoma prepared by immunization with HOCl-LDL, in which 2D10G9 (HOP-1) could react with HOCl-LDL and HOCl-BSA, also could recognize HOCl-modified (lipo)protein in human atherosclerotic lesions [24]; 10A7H9 could co-localize HOCl-modified proteins and myeloperoxidase in podocytes and adjacent basement membranes of human membranous glomerulonephritis [25]. Both mAb 2D10G9 and 10A7H9 were not used in immunohistochemistry staining on the sections from patients with diabetic nephropathy and IgA nephropathy. Also, the biological activities of these two mAbs were not reported.

In addition to recognition on AOPP presented in serum and tissue, we found that mAb 3F2 also can block the biological activity of AOPP (Figure 6). The result intensively indicates that the specific mAb may be a potential neutralizing agent for the research of AOPP pathology.

In conclusion, we generated a specific monoclonal antibody, 3F2, which reacts with AOPP in the serum and renal tissues from CKD patients and recognizes the accumulation of AOPP in the renal tissues of rats overloaded with AOPP. Moreover, 3F2 can inhibit the generation of ROS induced by AOPP. The immunoassays based on mAb 3F2 can be established for clinical evaluation of AOPP and for research on the pathogenesis of chronic kidney diseases.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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